New data on the structure and the chondrocyte populations of the haemal cartilage of abdominal vertebrae in the adult carp *Cyprinus carpio* (Ostariophysii, Cyprinidae)

by

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ABSTRACT. - The bulk of cartilage retained in the haemal arch of the abdominal vertebrae of adult carps was examined using scanning and transmission electron microscopy. A special attention was devoted to the mineralization of the cartilage. The mineralization is spheritic but the processes differ in the proximal and distal parts. The proximal hyaline cartilage contacts the lamellar bone of the centrum without an intermediate chondroid bone; it mineralizes although chondrocytes bearing a hypertrophic phenotype were not identified in this area. The distal part of the cartilage contains hypertrophic chondrocytes associated with the mineralization of the cartilage matrix. Mineralized globules fuse to form longitudinal septa that are replaced by trabecular bone after their resorption by multinucleated chondroclasts. The distal part, resembling a mammalian epiphyseal growth plate, was coined the "haemal growth plate". Two types of chondrocytes are identified in the distal part by transmission electron microscopy. Apart from typical chondrocytes called "light chondrocytes", a population of "dark chondrocytes" is present in all stages of chondrocyte differentiation. The dark chondrocytes are electron dense and they are characterised by an abundant rough endoplasmic reticulum and by Golgi complexes. Their nuclei show irregular outlines and they contain a very-condensed chromatin. The compacted chromatin is scattered within the nuclei. Some dark chondrocytes show signs of shrinking and self-destruction through autophagic vacuoles and blebbing with disruption of the cellular membrane. Such chondrocytes, considered as cells exhibiting chondroptosis, a variety of programmed cell death, have only been described so far in the growth plate of amniotes. In this study, dark chondrocytes following a chondroptotic pathway are described for the first time in the cartilage of a non-amniote vertebrate. The present study raises the possibility that specific microenvironmental conditions in the abdominal vertebrae of the adult carp prompt differentiation of dark cells.

RÉSUMÉ. - Nouvelles données sur la structure et les populations de chondrocytes dans le cartilage hémal des vertèbres abdominales de la carpe adulte *Cyprinus carpio* (Ostariophysii, Cyprinidae).

Le massif de cartilage qui persiste dans l'arc hémal des vertèbres abdominales chez la carpe adulte a été examiné à l'aide des microscopes électroniques à balayage et à transmission. Une attention toute spéciale a été portée aux modes de minéralisation du cartilage. La minéralisation est toujours de type sphéritique, mais ses processus diffèrent entre les régions proximale et distale. Le cartilage hyalin proximal est au contact de l'os lamellaire du centrum vertébral sans couche intermédiaire d'os chondroïde; il se minéralise bien qu'aucun chondrocyte au phénotype hypertrophié n'ait été identifié dans cette partie proximale du cartilage hyalin ou à proximité. La partie distale du cartilage hémal contient des chondrocytes hypertrophiés associés à la minéralisation de la matrice. Les globules minéralisés fusionnent pour former des septa longitudinaux qui, après leur destruction par des chondroclastes multinucléés, sont remplacés par des travées d'os enchondral. Cette partie distale du cartilage hémal qui ressemble au cartilage de croissance épiphysaire des mammifères est appelée "cartilage de croissance hémal". Deux types de chondrocytes ont été identifiés à l'aide du microscope électronique à transmission. À côté de chondrocytes "normaux", appelés "chondrocytes clairs", des "chondrocytes sombres" sont présents à tous les stades de différenciation des chondrocytes. Les chondrocytes sombres sont caractérisés par un développement important du réticulum endoplasmique granuleux et des aires golgiennes. Leurs noyaux, à l'aspect contourné, contiennent une chromatine très dense, dispersée dans l'espace nucléaire. Les chondrocytes sombres diminuent de volume et ils émettent des vacuoles autophagiques et des gonflements de la membrane cellulaire qui provoquent sa rupture et causent l'autodestruction de la cellule. Ces chondrocytes sombres sont engagés dans la voie de la chondroptose, une variété de mort cellulaire. La présence de tels chondrocytes sombres n'a été mentionnée que dans le cartilage de conjugaison des os longs d'amniotes. Des chondrocytes sombres présentant un processus chondroptotique sont donc décrits pour la première fois dans le cartilage d'un vertébré non-amniote. L'éventualité d'une relation entre la différentiation de cellules sombres et des conditions microenvironnementales particulières dans les vertèbres abdominales de carpe est envisagée au cours de ce travail.

Key words. - Cyprinidae - *Cyprinus carpio* - Carp - Haemal cartilage - Chondroptosis - Dark chondrocytes - Mineralization - Ultrastructure.

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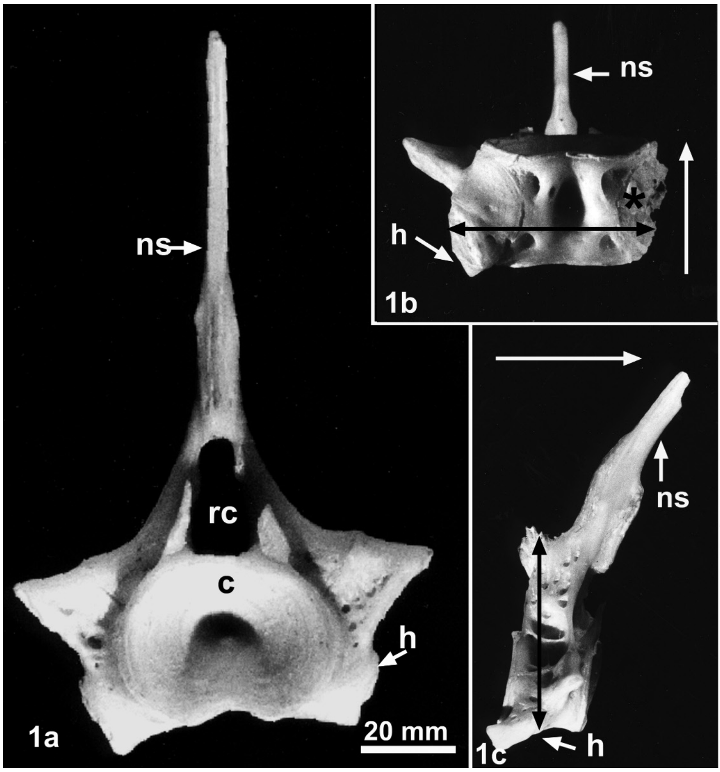
Most bones of the endoskeleton first appear as cartilaginous structures that are then replaced by bone. Most of these cartilaginous structures have a transient phenotype: the chondrocytes proliferate, then enlarge into hypertrophic chondrocytes, die and are replaced by osteoblasts during endochondral ossification. However, some – not joint-related – cartilaginous structures persist throughout life in mammals (Kimpel *et al*., 1999; Pufe *et al*., 2004; Kampen *et al*., 1995). In teleosts, the presence of a bulk of haemal cartilage in the haemapophysis of the abdominal vertebrae of adult carps (*Cyprinus carpio*) was first reported by Stephan (1900). In subsequent studies, as far as we know, it was confirmed that cartilaginous remnants at the vertebrae of adult teleosts were only observed in the haemal arches of the abdominal vertebrae of the carp (Hübner, 1961; Meunier and Boivin, 1974; Meunier, 1979). In the carp, as in other Cyprinidae, the haemal arches originate from two cartilaginous basiventralia. The basiventralia together with two basidorsalia that form the neural arch, compose the arcualia (François, 1966, 1967; Schaeffer, 1967). The cartilaginous arcualia differentiate from the perichordal mesenchyme during the development of the amphicoelous vertebrae in teleosts (Liem *et al*., 2001). In contrast, the vertebral bodies arise by intramembranous ossification with the fibrous notochord sheet as the first mineralized structure (Potthoff *et al*., 1986; Grotmol *et al*., 2003, 2005; Fleming *et al*., 2004; Nordvik *et al*., 2005, Nordvik, 2007). The cartilaginous preformed arches are covered by a thin layer of bone that is formed by direct mineralization of the perichordal mesenchyme; they compose the arcocentrum (for review see François, 1967; Arratia *et al*., 2001; Nordvik *et al*., 2005; Nordvik, 2007).

The cartilaginous preformed arches exhibit variations in



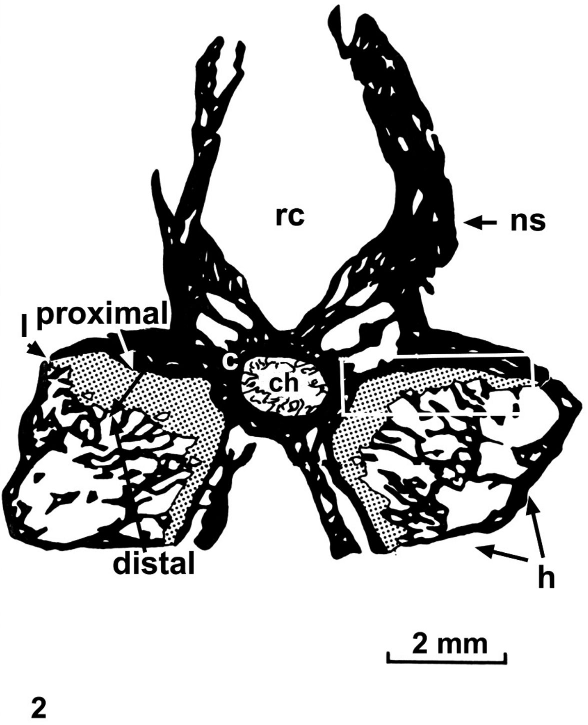
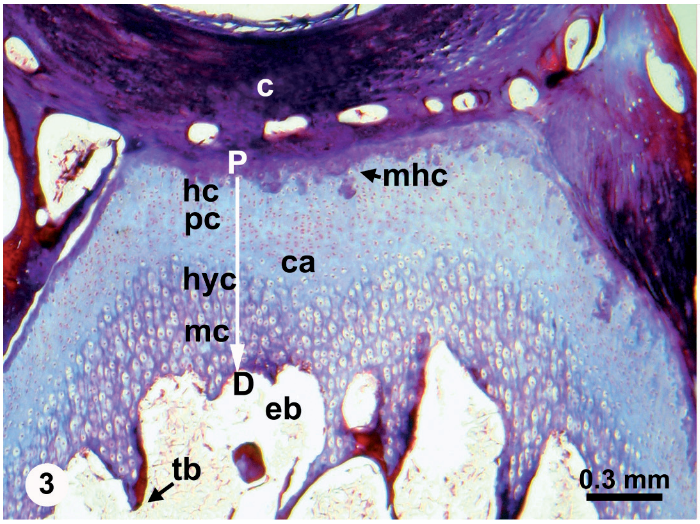
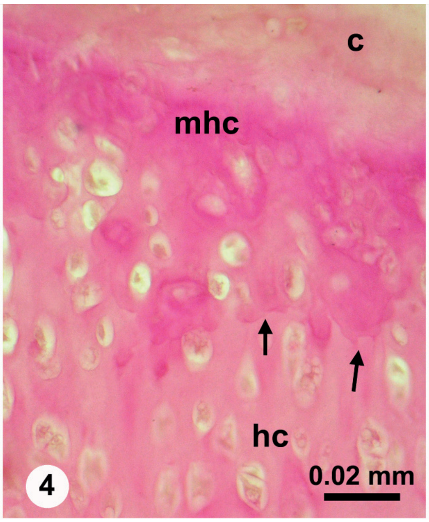


Figure 1. - Abdominal vertebra (V7). 1a. Anterior view. 1b. Ventral view. 1c. Lateral view. c: centrum ; h: haemapophysis; ns: neural spine; rc: rachidian canal. 1b, c. The anterior-posterior axis of the body is indicated by the long white arrows. The double-headed black arrows show the level of the cross sections used for light microscopy and TEM studies. The asterisk shows the location of the haemal cartilage after the removing of the external part of the haemapophysis. *[Vertèbre abdominale (V7). 1a. Vue frontale de la face antérieure. 1b. Vue ventrale. 1c. Vue latérale. c: centrum; h: hémapophyse; ns: épine neurale; rc: canal rachidien;. 1b,c. L'axe antéro-postérieur du corps est indiqué par les longues flèches blanches. Les flèches noires à doubles têtes indiquent le niveau des coupes transversales utilisées pour les observations en microscopie photonique et en microscopie électronique à transmission (MET). L'astérisque montre le cartilage hémal après l'ablation de la partie superficielle de l'hémapophyse.]*

Figure 2. - Schematic drawing of a cross section of an abdominal vertebra at the level of the haemapophysis. Black area: bone; stippled area: cartilage ; c: centrum ; ch: notochordal remnant; h: haemapophysis; l: ligament; ns: neural spine; rc: rachidian canal. The insert represents the area examined in TEM. *[Schéma d'une coupe transversale d'une vertèbre abdominale au niveau de l'hémapophyse. L'os est figuré en noir, le cartilage en pointillé. c: centrum; ch: résidu chordal, h:hémapophyse, l: ligament; ns: épine neurale; rc: canal rachidien. La partie encadrée représente la surface examinée en MET.]*





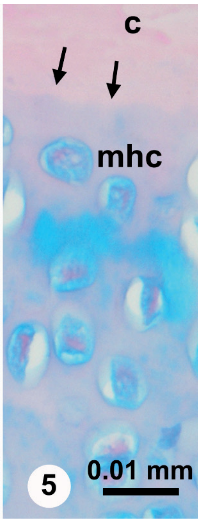


Figure 3. - Cross-section of an abdominal vertebra. One-step trichrome. The centrum (c) is composed of lamellar bone. The osseous tissue contacts a thin layer of mineralized hyaline cartilage (mhc). Under the mineralized layer, the cartilage is composed of the following layers from the proximal (P) to the distal part (D) (white arrow): hyaline cartilage (hc); proliferative cartilage (pc); hypertrophic cartilage (hyc); distal mineralized cartilage (mc). Erosive bays (eb) are observed at the distal part of the haemal cartilage where the mineralized cartilage is in contact with trabecular bone (tb). *[Coupe transversale d'une vertèbre abdominale. Trichrome en un temps. Le centrum (c) est constitué d'os lamellaire. Le tissu osseux est au contact de la fine couche de cartilage hyalin minéralisé (mhc). Au-dessous de cette couche minéralisée, le cartilage est composé des couches suivantes de la partie proximale (P) vers la partie distale (D) (flèche blanche): cartilage hyalin (hc) ; zone de prolifération (pc) ; cartilage hypertrophique (hyc) ; cartilage distal minéralisé (mc). Des baies d'érosion (eb) sont observées dans la partie distale du cartilage hémal où le cartilage minéralisé est au contact de travées osseuses (tb).]*

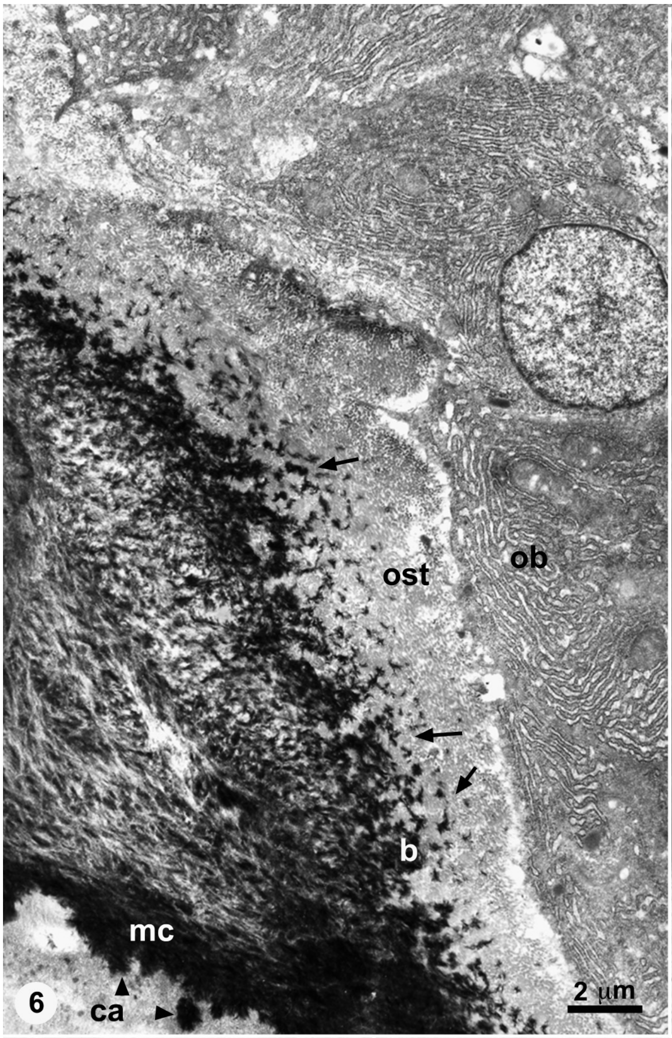
Figure 4. - Detail of the contact between the centrum and the proximal haemal cartilage. PAS-Groat's haematoxyline. The mineralized hyaline cartilage (mhc) is more PAS-reactive than the centrum osseous tissue (c) and the unmineralized hyaline cartilage (hc). Mineralized globules ornament the inner surface of the mineralized hyaline cartilage (arrows). *[Détail de la zone de contact entre le centrum et la partie proximale du cartilage hémal. APS-hématoxyline de Groat. Le cartilage hyalin minéralisé (mhc) est plus intensément APS-positif que le tissu osseux du centrum (c) et que le cartilage hyalin non minéralisé. Des globules minéralisés sont visibles en surface du cartilage hyalin minéralisé (flèches).]*

Figure 5. - Same area as in figure 4. Alcian blue pH 2.5-nuclear solid red. A very light staining is observed in the mineralized cartilage (arrows). Below, acid mucosubstances are found in the unmineralized cartilage. The centrum osseous tissue is not reactive. *[Même zone que figure 4. Bleu alcian pH 2,5- rouge nucléaire solide. La partie minéralisée du cartilage hyalin (mhc) est légèrement colorée par le bleu alcian (flèches). Des mucosubstances acides sont présentes dans la partie non minéralisée du cartilage. Le tissu osseux du centrum (c) n'est pas coloré par le bleu alcian.]*

development, morphology and ossification depending on the position of the vertebra along the vertebral column and the phylogenetic position of the species (François, 1967, Bird and Mabee, 2003). Cyprinidae are considered to belong to less advanced taxa of teleosts, together with Clupeidae and Salmonidae (Arratia, 1997), partly because the cartilage of the arcualia is maintained for a longer period of time (François, 1966; Kranenbarg *et al*. 2005) than in Paracantopterygii and Acantopterygii. For example, cartilage is not observed in early development of arcualia of Gadidae and Gobiidae that mineralize through direct ossification (François, 1966, 1967). The persistence of cartilage in the basiventralia at the carp abdominal vertebrae in adults prompted us to obtain detailed insights into the processes of mineralization in late developmental stages and in adults. Few previous studies concern the differentiation and the mineralization of these cartilaginous remnants (Meunier and Boivin, 1974; Meunier, 1979).

MATERIAL AND METHODS

For the present study, vertebrae of juvenile and adult carps were removed from 5 specimens (total length from 200 mm to 470 mm) commercially obtained. For light microscopy, the vertebrae were fixed in a Bouin's mixture, dehydrated in butanol, and embedded in paraffin. Transversal and sagittal sections were stained with a one-step trichrome, Azan trichrome, and mucosubstances were identified with PAS and alcian blue at pH 0.5 and pH 2.5 (Gabe, 1968). Scanning electron microscopy (SEM) was carried out on vertebrae fixed in 70% ethanol. The haemapophyses were broken and immersed in a 12% sodium hypochlorite solution at room temperature to remove the unmineralized structures and to obtain cleaned mineralized fronts. The samples were then washed in distilled water, dehydrated in 100% ethanol, air-dried, glued and coated with evaporated gold. The surfaces of the calcified cartilage were observed in a Jeol-SEM-35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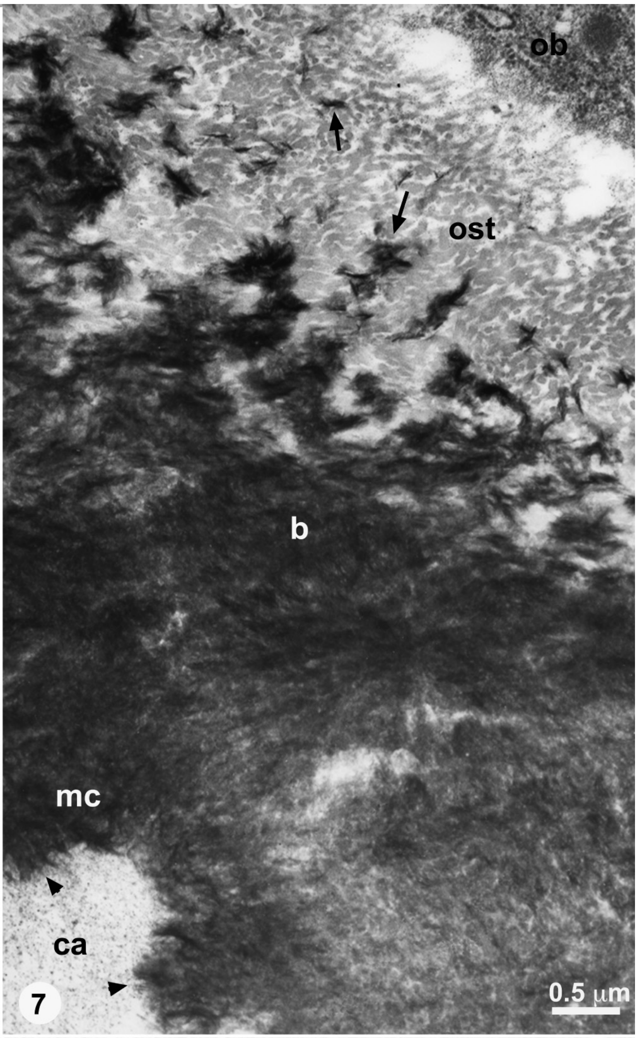
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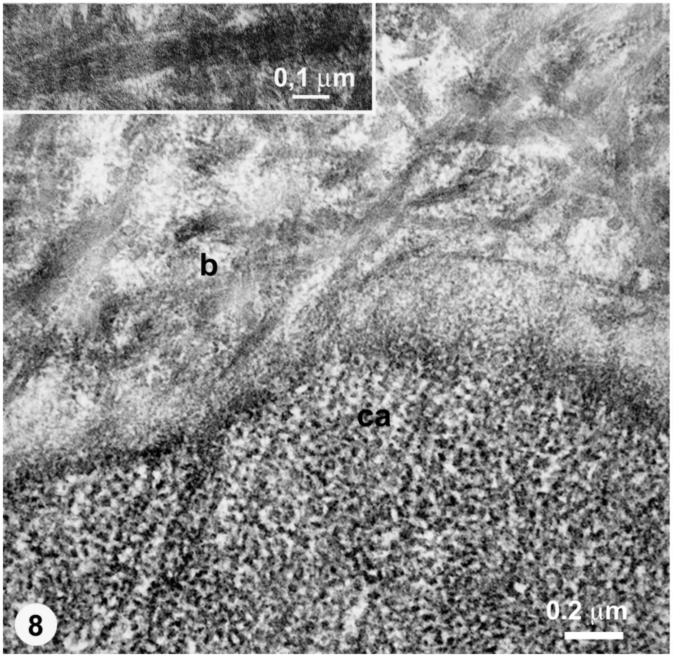
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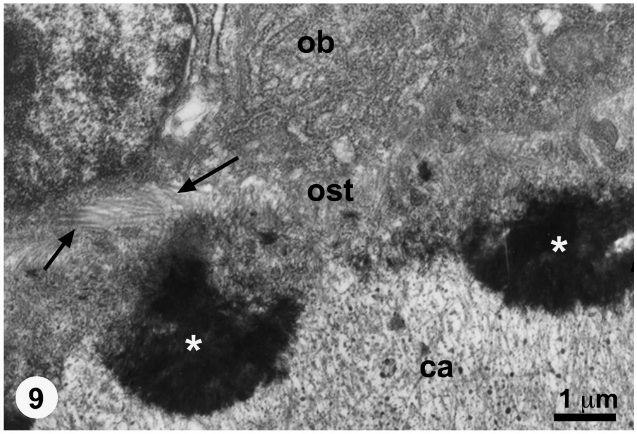
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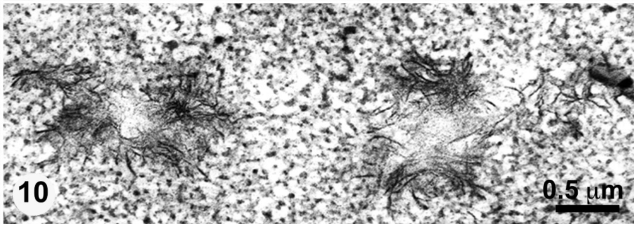
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scanning electron microscope at 25 kV. For transmission electron microscopy (TEM), small pieces of haemapophysis were fixed in a mixture containing 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M cacodylate buffer pH 7.4. Some samples were partially or entirely demineralized with 5% EDTA added in the fixative. Both undemineralized and demineralized samples were post-fixed with 1% osmium tetroxide in the cacodylate buffer, dehydrated then embedded in Epon. Semi-thin sections (1 μm thick) stained with buffered toluidine blue (pH 4), were examined by light microscopy to select appropriate areas for TEM examination. Thin sections were double stained with uranyl acetate and lead citrate, and viewed in a Philips 201 transmission electron microscope with an operating voltage 80 KV.

RESULTS

Review of the structure of abdominal vertebrae of adult carps

The abdominal amphicoelous vertebra of an adult carp is composed of an ossified centrum surrounding the notochordal remnant, an ossified dorsal arch from which a long ossified neural spine projects and the two symmetrical haemapophyses forming a ventral incomplete haemal arch (Fig. 1). The haemal arch contains cartilage tissue in the shape of a cup that is sandwiched between two layers of osseous tissues. The two halves of the haemal arch are linked by a partially mineralized ligament (Fig. 2). Proximal, the haemal cartilage connects to the vertebral centrum, made of lamellar bone. Distal, it contacts the trabecular bone of the arcocentrum (Fig. 2). Transverse histological sections of the haemapophyses show in the proximal part a thin layer of mineralized hyaline cartilage (Fig. 3). Below this proximal mineralized cartilage layer, the haemal cartilage is composed of superimposed zones resembling those described in mammalian epiphyseal growth plates (for review, see Karaplis, 2002): it consists in a hyaline zone, a proliferative zone, a hypertrophic zone, a mineralized cartilage zone, and a bone formation zone (Fig. 3). Therefore, we propose to call this distal part “haemal growth plate” (HGP).

The mineralization of the proximal and the distal parts of the haemal cartilage undergoes different processes that are described in the next paragraphs.

Proximal part of the haemal cartilage

Histology

The thin mineralized layer abuts the bone of the centrum. Its distal side is ornamented with thin and distally orientated mineralized extensions (Figs 3, 4).

This layer shows a stronger PAS-reaction than the bone of the centrum (Fig. 4). Acidic mucosubstances reactive to alcian blue are present only in the cartilage (Fig. 5).

Figures 6-10.
Proximal area of the cartilage of the haemal growth plate. TEM. Unless indicated all sections for TEM are from undemineralized samples. *[Partie proximale du cartilage de l’hémapophyse. MET. En l’absence d’indication particulière, les coupes utilisées pour la MET proviennent d’échantillons non déminéralisés.]*

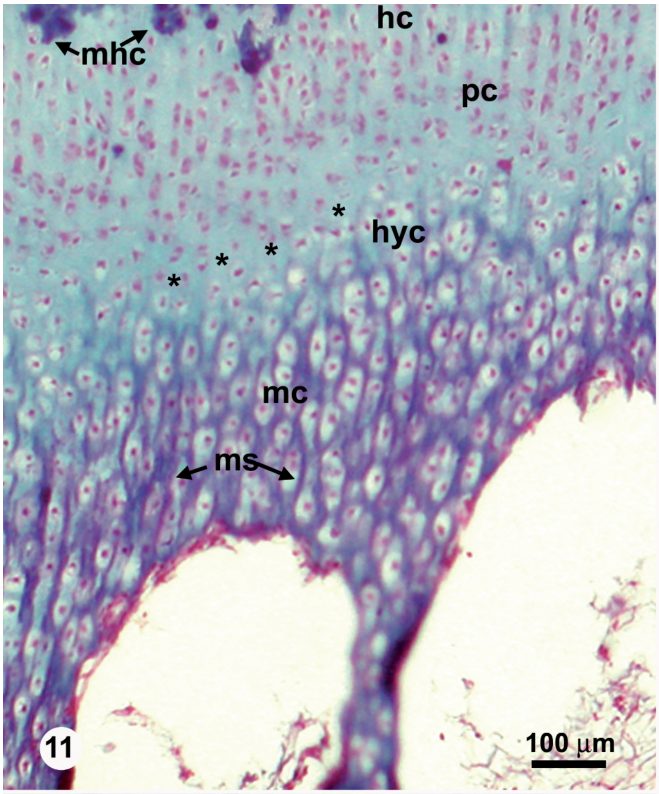
Figure 6. - Partially demineralized section. Contact between the osseous tissue of the centrum and the hyaline cartilage in the lateral area of the haemapophysis near the ligament (l in figure 2). On the bone side, osteoblasts (ob) show a well-developed RER and Golgi areas. In the osteoid area (ost), crystallites (arrows) are associated with the collagen fibrils. In the mineralized cartilage (mc), the crystallites fuse to form globules (arrowheads). b: bone; ca: cartilage. *[Coupe partiellement déminéralisée. Zone de contact entre le tissu osseux formant le centrum et le cartilage hyalin de la partie latérale de l’hémapophyse proche du ligament (l dans la figure 2). Du côté osseux, les ostéoblastes (ob) montrent un RER et des aires golgiennes bien développés. Dans la zone ostéoïde (ost), les cristaux (flèches) sont associés aux fibrilles de collagène. Dans le cartilage calcifié (mc), les cristaux s’agglutinent pour former des globules (têtes de flèches). b : tissu osseux; ca : cartilage.]*

Figure 7. - Detail of the contact between bone and cartilage. In the osteoid area (ost), the crystallites are closely associated with the collagen fibrils (arrows). In the mineralization front of cartilage (ca), the crystallites (arrowheads) form dense clusters. b: bone; mc: mineralized cartilage; ob: osteoblast. *[Détail de la zone de contact entre le tissu osseux du centrum et le cartilage. Dans la zone ostéoïde (ost), les cristaux sont étroitement associés aux fibrilles de collagène (flèches). Au niveau du front de minéralisation du cartilage, les cristaux (têtes de flèches) forment des amas denses. b : tissu osseux ; mc : cartilage minéralisé ; ob : ostéoblaste.]*

Figure 8. - Section of a demineralized sample showing a direct contact between cartilage (ca) and bone (b) that contains thick collagen fibrils. Insert : Detail of a bone collagen fibril showing the characteristic periodic banding. *[Coupe d’un échantillon déminéralisé montrant le contact entre le cartilage (ca) et l’os (b) qui contient des fibrilles de collagène de fort calibre. Encart : Détail d’une fibrille de collagène du tissu osseux avec sa striation périodique caractéristique.]*

Figure 9. - Mineral deposit in the proximal hyaline cartilage. Note the presence of thick collagen fibrils (arrows) forming the osteoid zone (ost) between the osteoblast (ob) and the mineralized cartilage (asterisks). ca: cartilage. *[Dépôt minéral dans le cartilage hyalin proximal. Noter la présence de fibrilles de collagène (flèches) de fort calibre dans la zone ostéoïde (ost) située entre les ostéoblastes (ob) et le cartilage minéralisé (astérisques). ca : cartilage.]*

Figure 10. - Clusters of mineral crystals within the hyaline cartilage. *[Groupes de cristaux dans le cartilage hyalin.]*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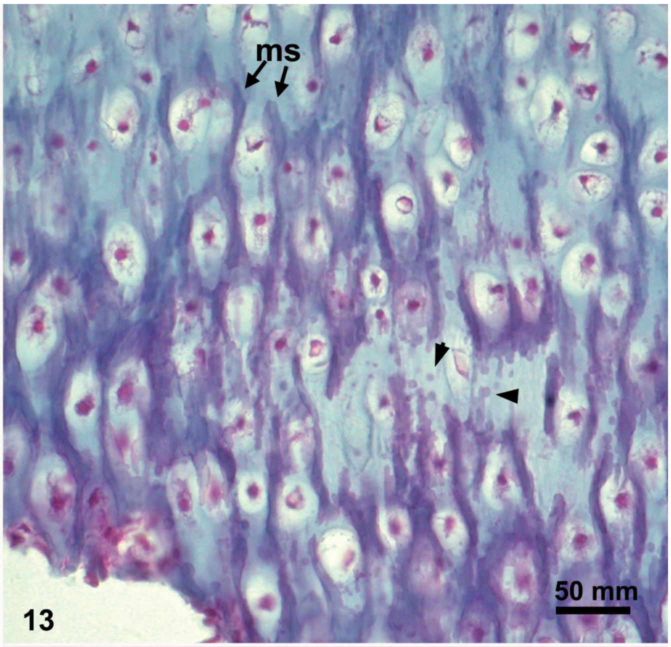
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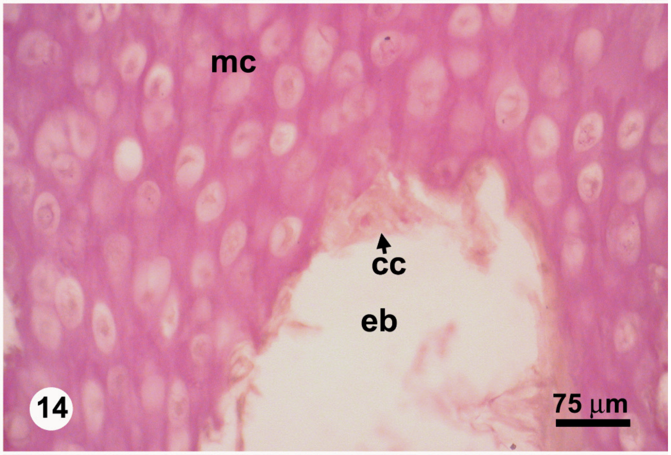
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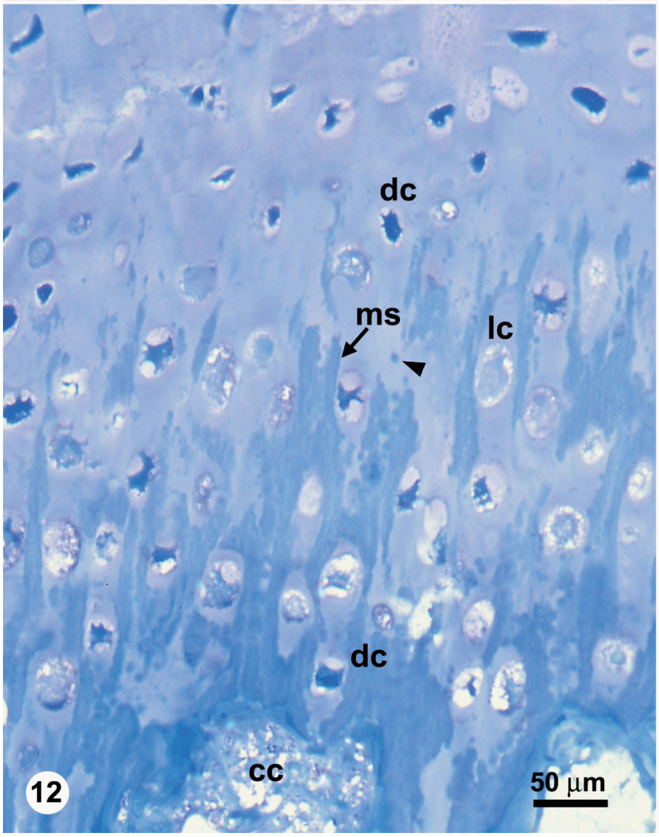
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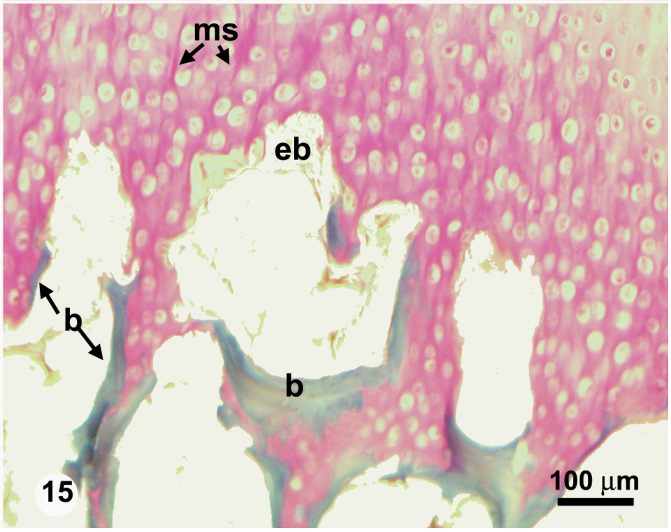
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Ultrastructure

The proximal mineralized cartilage appears at the ultrastructural level to be composed of cartilage and bone (Fig. 6). In the lateral part of the haemapophysis (Fig. 2) where ossification proceeds, osteoblasts located on the surface of the forming bone show well-developed saccules of rough endoplasmic reticulum (RER) and Golgi areas, both reflecting a high synthetic activity in these cells. Osteoblasts produce striated collagen fibrils of about 100 nm in diameter resembling type I collagen fibrils characteristic of the osseous tissues (Fig. 6). Unmineralized collagen fibrils form an osteoid layer in the vicinity of the osteoblasts. The mineralization starts by the deposit of individual crystals oriented by and along the collagen fibrils (Fig. 7), then the crystals invade the fibrils and the whole space (Fig. 7). On the side of the mineralized layer that faces the haemal cartilage, mineralized globules emerge from the thin layer of mineralized cartilage whose limits with the osseous tissue are not distinguishable on mineralized thin sections (Figs 6, 7). The boundary between the osseous tissue and the mineralized cartilage is obviously observed in thin sections of demineralized specimens (Fig. 8). The thick striated collagen fibrils of the osseous tissue appear in contact with thin fibrils resembling type II collagen fibrils associated with abundant granules, most probably aggrecan granules, both being characteristic of the cartilage matrix. Thick and thin collagen fibrils do not intermix (Fig. 8). The mineralization of the cartilage layer starts with the deposit of thin crystals not bound to the collagen fibrils, but arranged in individual clusters according to spheritic mineralization processes (Ørvig, 1951, 1968) (Fig. 9). The clusters fuse to form globules. Then, individual globules also fuse with one another and with the mineralized osseous layer deposited in the osteoid zone. The latter form a narrow space between the mineralized layer and the osteoblasts (Fig. 9). Mineralization of the proximal cartilage layer was observed in an area where the globular chondrocytes do not show the characteristics of hypertrophic chondrocytes seen in the distal part of the cartilage where the haemal growth plate differentiates.

Distal part of the haemal cartilage

Histology

Below the thin mineralized hyaline cartilage of the proximal part of the carp haemal cartilage, the HGP is composed of the following layers (Fig. 11). 1] A very thin hyaline zone that corresponds to a resting zone where cells appear spherical in cross section and randomly distributed within an abundant extracellular matrix (ECM) stained green with one-step trichrome (Figs 3, 11) or blue with azan (not shown). 2] A

Figures 11-15.
Distal cartilage. Haemal growth plate. Light microscope. *[Partie distale du cartilage. Plaque de croissance hémale. Microscopie photonique.]*

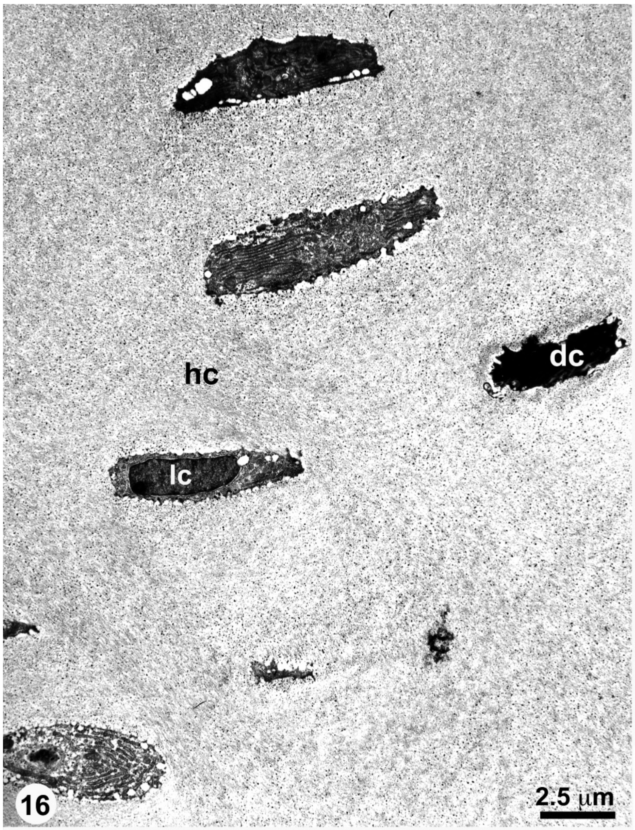
Figure 11. - One-step trichrome. On the top of the figure, mineralized globules (arrows) at the front of mineralization of hyaline cartilage (mhc). The chrondrocytes of the proliferative zone are distributed in parallel columns (pc) below the unmineralized hyaline cartilage (hc). In the distal mineralized cartilage (mc), mineralized septa (ms) encircle the columns of hypertrophic chondrocytes (hyc). The separation between the proliferative zone and the hypertrophic zone is obvious (asterisks). *[Détail de l'organisation du cartilage hémal. En haut de la figure, des globules minéralisés (flèches) sont situés au front de minéralisation du cartilage hyalin (mhc). Sous la couche de cartilage hyalin non minéralisé (hc), les chondrocytes de la zone de prolifération forment des colonnes parallèles (pc). Dans le cartilage minéralisé de la partie distale (mc), des septa minéralisés (ms) séparent les colonnes de chondrocytes hypertrophiés (hyc). La limite entre les zones de prolifération et le cartilage hypertrophié est très nette (astérisques).]*

Figure 12. - Semi-thin section. Toluidine blue. Dark chondrocytes (dc) strongly stained are distributed in the whole HGP. Mineralized longitudinal septa (ms) surround light (lc) or dense (dc) chondrocytes. Mineralized globules are present in the vicinity of the septa (arrowhead). Clastic cells (cc) are present at the distal part of the mineralized cartilage. *[Coupe semi-fine. Bleu de toluidine. Des chondrocytes sombres (dc) intensément colorés sont répartis dans toute la plaque de croissance. Des septa longitudinaux minéralisés (ms) enserrent les chondrocytes clairs (lc) ou denses (dc). Des globules minéralisés sont au voisinage des septa (tête de flèche). Des cellules clastiques (cc) se trouvent au contact de la partie distale du cartilage minéralisé.]*

Figure 13. - Distal part of the mineralized cartilage. One-step trichrome. Hypertrophic chondrocytes are housed in capsules surrounded by the mineralized septa (ms). Mineralized globules (arrowheads) are isolated or fuse with the septa. *[Partie distale du cartilage minéralisé. Trichrome en un temps. Les chondrocytes hypertrophiques sont logés dans des capsules entourées par les septa minéralisés (ms). Des globules minéralisés sont isolés ou se soudent aux septa (têtes de flèches).]*

Figure 14. - Detail of the distal part of the mineralized cartilage. PAS-Groat haematoxyline. The mineralized septa are PAS-positive. In the erosive bay (eb), a multinucleated chondroclast (cc) is located at the distal part of the mineralized cartilage (mc). *[Détail de la partie distale du cartilage minéralisé. APS-hématoxyline de Groat. Les septa sont colorés par l'APS. Baie d'érosion (eb) avec un chondroclaste multinucléé (cc) accolé à la partie distale du cartilage minéralisé (mc).]*

Figure 15. - Distal part of the haemal cartilage. PAS-Groat haematoxyline-picroindigocarmine. The mineralized cartilage lining the erosive bays (eb) is associated with osseous tissue (b). ms: mineralized septa. *[Partie distale. APS-hématoxyline de Groat-picroindigocarmin. Le cartilage minéralisé comporte des baies d'érosion (eb) où il est au contact du tissu osseux (b). ms : septa minéralisés.]*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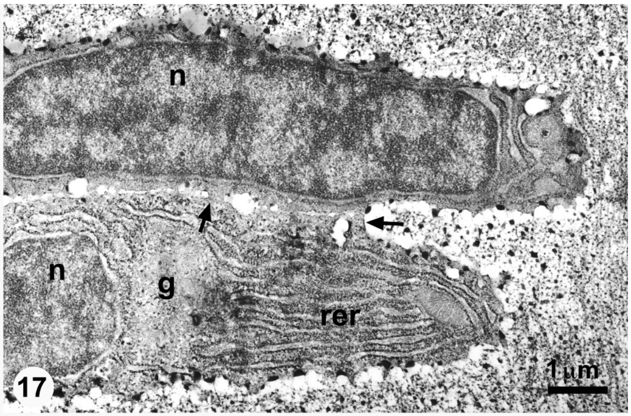
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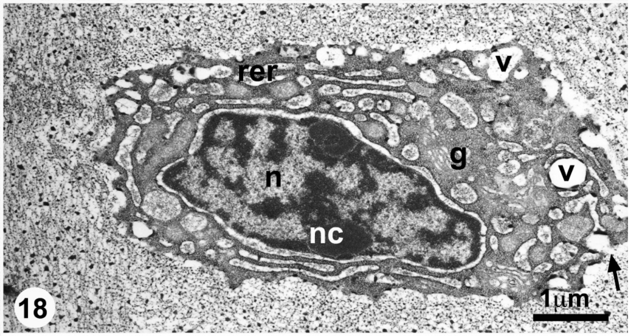
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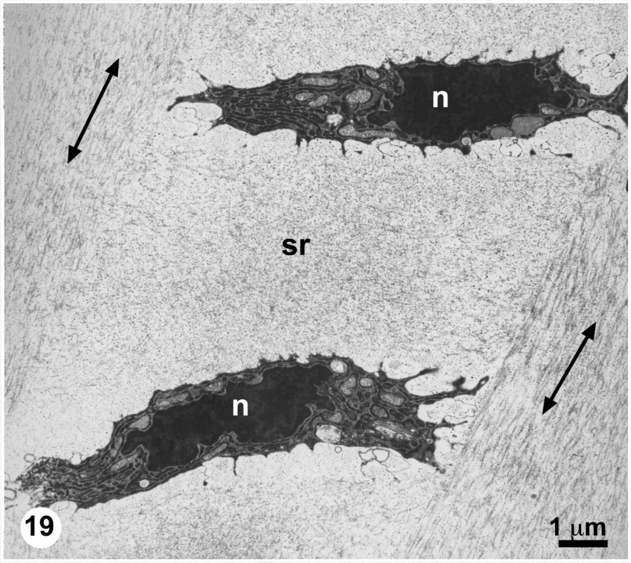
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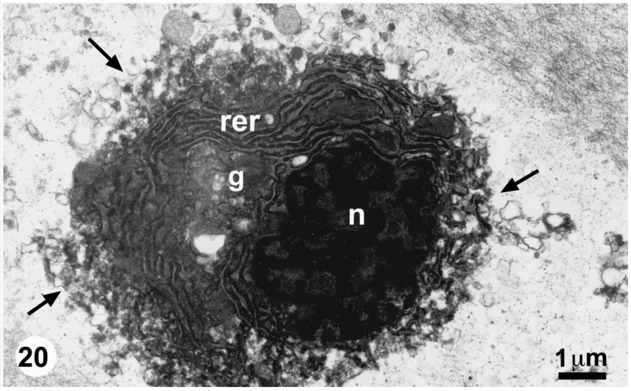
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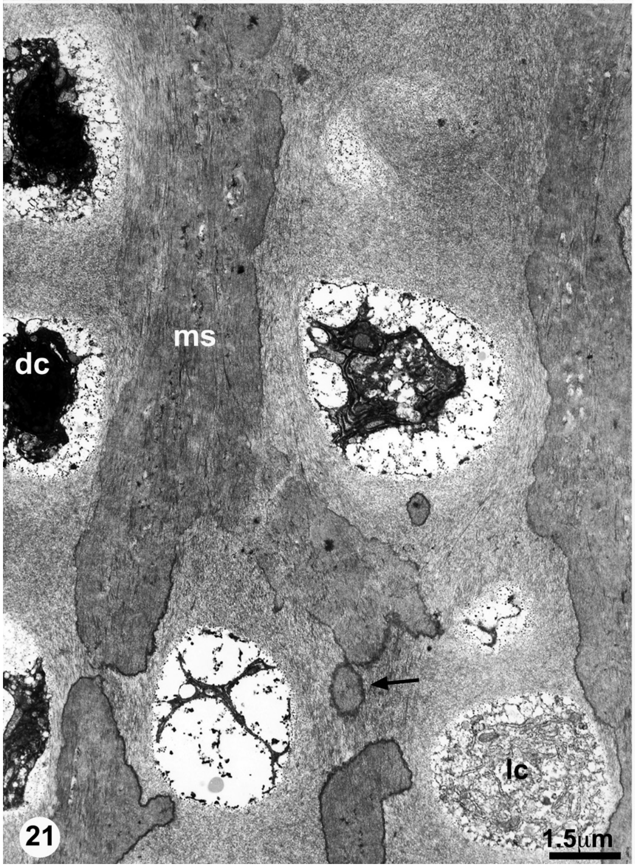
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proliferative zone where chondrocytes become flat cells and divide. The daughter cells remain close to one another forming isogenic groups. The groups are surrounded by the extracellular matrix, and they are arranged in approximately parallel columns (Figs 11-13). 3] A hypertrophic zone. The chondrocytes loose their discoid shape and start to enlarge. Thus, an obvious decrease of the extracellular matrix takes place concomitantly with the enlargement of the hypertrophic chondrocytes (Fig. 11). Semi-thin sections stained with toluidine blue reveal the presence of two populations of chondrocytes: one stained in light blue, and the other in dark blue (Fig. 12). Longitudinal septa mineralize in the distal part of the hypertrophic zone. They separate more randomly organised columns where each chondrocyte is housed in a large lacuna, the capsule (Figs 12-15). One-step trichrome stains purple the mineralized septa, and granules distributed in the unmineralized matrix stained green (Fig. 13). The mineralized septa are more intensively stained with toluidine blue in semi-thin sections (Fig. 12), and they are PAS-positive (Figs 14, 15). 4] Mineralized cartilage zone. The distal part of the haemal cartilage is invaded by vascular mesenchyme (Figs 11, 15). Multinucleated chondroclasts line the distal surface of the cartilage (Figs 12, 14). 5] Bone formation zone. The septa of mineralized cartilage are replaced by thin trabeculae of osseous tissue (Figs 3, 15).

The growth plate of the haemal arch shows a clear-cut separation between the proliferative and the hypertrophic zones (Figs 3, 11). Thus, it differs from an ephiphyseal growth plate of mammal long bone where the different zones are distinct, but merge gradually into each other.

Ultrastructure

The hyaline cartilage matrix is made of thin collagen fibrils associated with abundant aggrecan particles (Fig. 16). Consistent with light microscopic observations, TEM data reveal the presence of two types of chondrocytes in the different zones of the HGP (Fig. 16). One type corresponding to regular chondrocytes was termed “light chondrocytes” (Figs 16, 17) by comparison with the other type coined “dark chondrocytes” according to the mammalian and avian nomenclature of Wilsman *et al*. (1981), Farnum and Wilsman (1989) and Erenpreisa and Roach (1998) (Figs 16, 18-20). The dark chondrocytes of the HGP show abundant long saccules of RER that surround the nucleus and containing a meshwork of fine filaments; they have a well-developed Golgi apparatus; these cytological characteristics reflect an intense synthetic activity. A high number of vacuoles are distributed in the periphery of the cell, some of them being extruded into the extracellular space (Fig. 18). The dark chondrocytes are also characterised by the structure of their nuclei that contain a more condensed chromatin than the light chondrocytes. A well-developed nucleolus is often discernible (Fig. 18). In the proliferative zone, the flattened light chondrocytes appear like daughter cells forming isogenic groups (Fig. 17), the flattened dark chondrocytes also appear as daughter cells but show a more condensed chro-

Figures 16-21.
Distal cartilage of the haemal arch. TEM. *[Cartilage distal de l’hémapophyse. MET.]*

Figure 16. - Flattened dark chondrocytes (dc) and light chondrocytes (lc) are distributed within the hyaline cartilage (hc). *[Chondrocytes sombres (dc) et chondrocytes clairs (lc) aplatis, répartis dans la matrice du cartilage hyalin (hc).]*

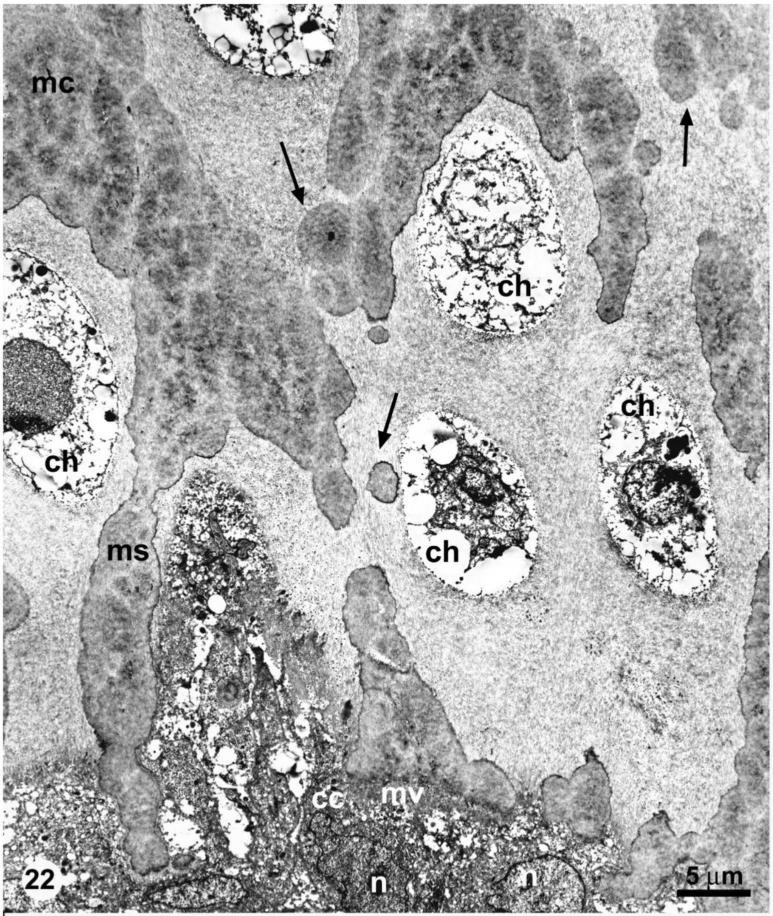
Figure 17. - Two flattened isogenic light chondrocytes connected by cytoplasmic bridges (arrows). g: Golgi area; n: nucleus; rer: RER. *[Groupe isogénique formés de deux chondrocytes “clairs” réunis par des ponts cytoplasmiques (flèches). g : aires golgiennes ; n : noyau ; rer : RER.]*

Figure 18. - Chondrocyte starting to differentiate into dark chondrocyte and containing vacuoles (v), a developed RER (rer) around the nucleus (n) and a Golgi area (g). nc: nucleolus. *[Chondrocyte se différenciant en chondrocyte “sombre”, contenant des vacuoles (v), un RER (rer) bien développé autour du noyau (n) et une aire golgienne (g). nc : nucléole.]*

Figure 19. - Two flattened dark chondrocytes with dark nuclei (n). The thin fibrils of the cartilage extracellular matrix are cross-sectioned in the transverse septum (sr) between two chondrocytes of the same column. They are longitudinally oriented (double-headed arrows) in the longitudinal septa surrounding the columns of chondrocytes. *[Deux chondrocytes “sombres” avec des noyaux sombres (n). Les fines fibrilles de la matrice extracellulaire du cartilage sont coupées tranversalement dans le septum transverse (sr) qui sépare deux chondrocytes de la même colonne. Ces fibrilles sont coupées longitudinalement dans les septa longitudinaux qui entourent les colonnes de chondrocytes (flèches à double tête).]*

Figure 20. - A globular dark chondrocyte showing a dark nucleus (n) surrounded by a developed RER (rer) and Golgi area (g). The cell membrane is disrupted by the extrusion of numerous vacuoles (arrows). *[Chondrocyte “sombre” avec un noyau (n) très opaque aux électrons, entouré par un RER (rer) bien développé et à proximité d’une aire golgienne (g). La membrane cellulaire est fragmentée avec extrusion de vacuoles (flèches).]*

Figure 21. - Demineralized section of the distal part of the hypertrophic cartilage. Encapsulated globular dark chondrocytes (dc) and light chondrocyte (lc) form columns separated by mineralized septa (ms). An isolated globule is attached to the distal end of a septum (arrow). *[Coupe déminéralisée de la partie distale du cartilage hypertrophié. Des chondrocytes “sombres” globuleux (dc) et des chondrocytes clairs (lc), logés dans des capsules, forment des colonnes séparées par des septa minéralisés (ms). Un globule isolé est attaché à la partie distale d’un septum (flèche).]*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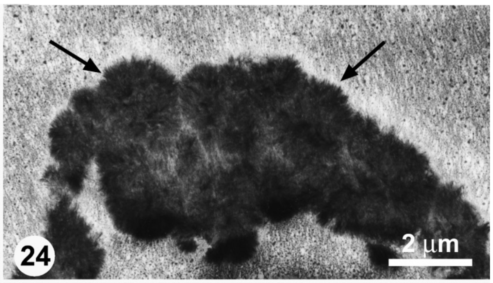
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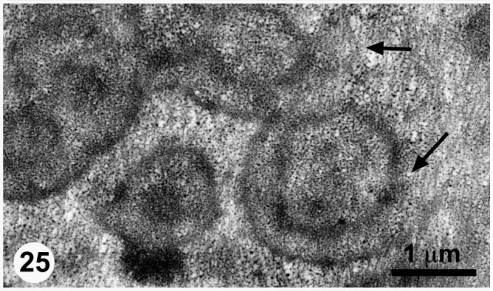
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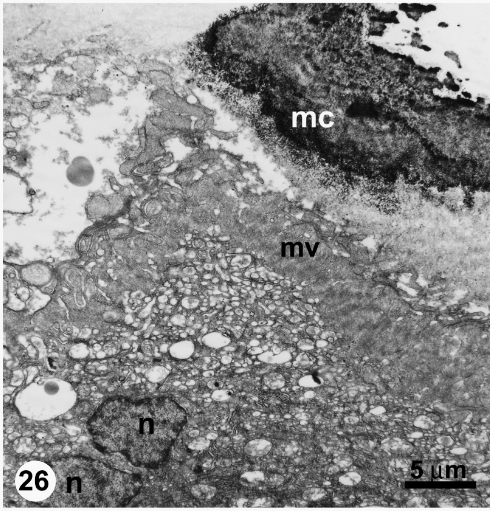
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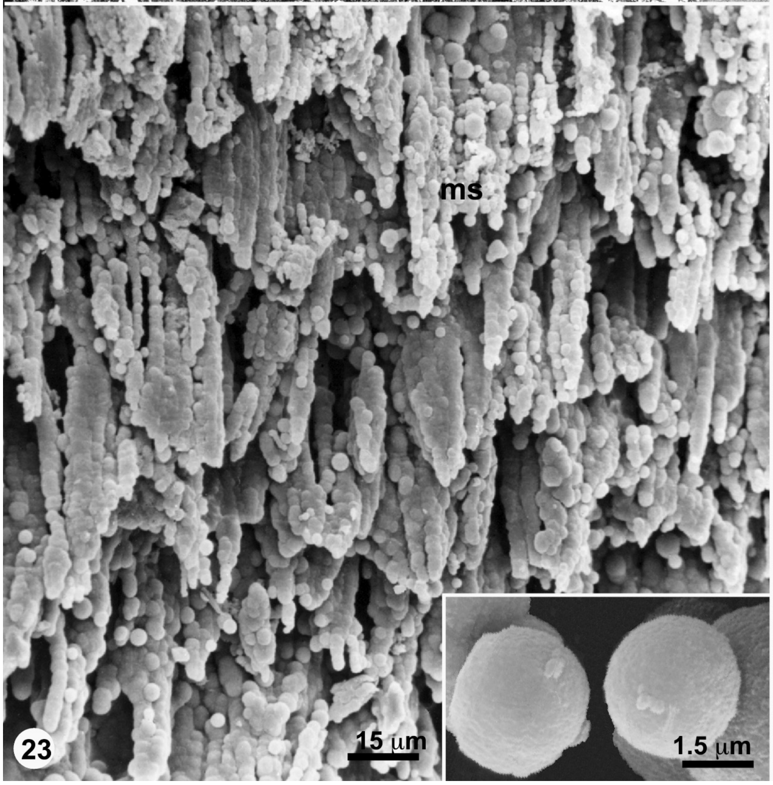
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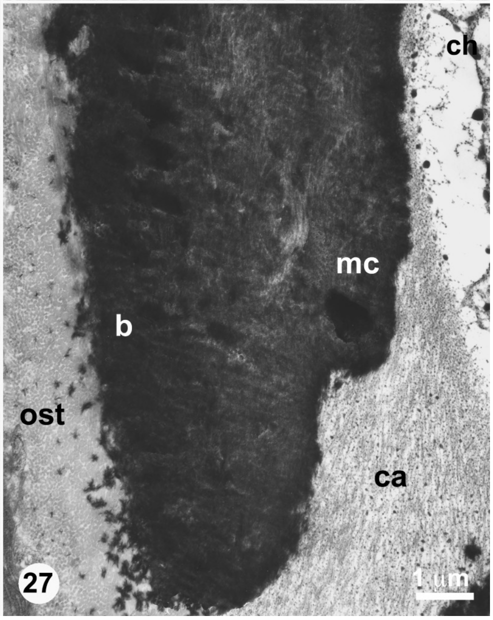
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matin (Fig. 19). When the chondrocytes are arranged in columns, the thin collagen fibrils are oriented in parallel within the septa. The longitudinally oriented fibrils of the longitudinal septa are perpendicular to those of the transverse septa (Fig. 19). Both light and dark chondrocytes change their shape from flattened to globular in the hypertrophic zone (Fig. 21). Each chondrocyte is housed within a capsule (Fig. 21). As light chondrocytes become hypertrophic, they increased their volume whilst dark chondrocytes undergo shrinking. They possess an indented nuclear envelope. The nuclei contain a very condensed chromatine, they become more and more electron-opaque. The nuclear material is scattered within the nucleus (Figs 19-21), even in the hypertrophic dark chondrocytes found in the very distal part of the HGP (Figs 20-22). The extensive RER is still present but it forms flat saccules. Blebbing and release of abundant vacuoles in the extracellular spaces are consistent with a disruption of the cell membrane that leads to a decrease of the cytoplasmic components by self-destruction (Fig. 20). In dark chondrocytes, autophagic vacuoles are responsible for cytoplasm degradation that is concomitant with a disruption of the nuclear membrane followed by the degradation of the nuclear material (Figs 21, 22). Cellular debris remains within the capsules in the mineralized zone (Figs 21, 22). Some capsules contain necrotic light chondrocytes (Fig. 21). The capsules are surrounded by the mineralized longitudinal septa; the transverse septa do not mineralize. The mineralized globules compose the longitudinal septa (Figs 22, 23). They are first isolated globules (Fig. 22) that fuse forming the "stalactites" observed in scanning electron micrographs of specimens where the unmineralized cartilage was removed by sodium hypochlorite (Fig. 23). The mineralized globules are formed through spheritic mineralization (Figs 24, 25). The globule size increases by deposition of concentric layers as observed on partially demineralized TEM sections where the globules show an electron dense central core surrounded by electron dense rings, the Liesegang's rings reflecting periodic mineral deposits (Fig. 25). Multinucleated chondroclasts adhere to the very distal parts of the mineralized septa. The chondroclasts are responsible for the destruction of the mineralized cartilage replaced by osseous tissue (Figs 14, 22, 26). They have a developed ruffled border region associated with the destruction of the mineralized cartilage (Fig. 26). Mineralized cartilage and osseous tissue are closely joined side by side (Figs 15, 27).

DISCUSSION

We present new data on two mineralization processes that progress at the two opposite regions of the haemal cartilage, a structure that persists in adult carps only in the abdominal haemal arches (Stephan, 1900; Meunier and Boivin, 1974; Meunier, 1979). The present ultrastructural study has shown that on its proximal side, the haemal cartilage remnants are contiguous with the lamellar bone of the centrum. However, an obvious boundary is observed between the osseous tissue and the mineralized cartilage. The mineralizing processes in this area are involved in the formation of a mineralized cartilage layer whose mineraliza-

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Figures 22-27.
Distal part of the haemal arch growth plate. *[Partie distale de la plaque de croissance de l'arc hémal.]*

Figure 22. - TEM. Demineralized section. A chondroclast (cc) with two nuclei (n) is attached to the distal part of a septum (ms). Chondrocytes (ch) located within capsulae show various degenerative aspects. Mineralized globules are attached or located close to the septa (arrows). mc: mineralized cartilage ; mv: microvilli. *[MET. Coupe déminéralisée. Un chondroclaste (cc) possédant deux noyaux (n) est accolé à la partie distale d'un septum (ms). Des chondrocytes (ch) situés dans des logettes chondrocytaires montrent des aspects variés de dégénérescence. Des globules minéralisés sont attachés ou sont proches des septa (flèches). mc : cartilage minéralisé ; mv : microvillosités.]*

Figure 23. - SEM. General view of the very distal part of the hypertrophic cartilage. The unmineralized tissues have been removed. Numerous mineralized globules form the septa (ms) that appear like pillars surrounding empty spaces where the unmineralized material was housed (extracellular matrix and hypertrophic chondrocytes). Insert: Detail of two mineralized globules. *[MEB. Vue générale de la partie distale du cartilage hypertrophié. Les tissus non minéralisés ont été détruits. Septa (ms) composés de globules minéralisés qui forment des piliers autour d'espaces vides occupés par la matrice extracellulaire non minéralisée et par les chondrocytes hypertrophiés. Encart : détail de deux globules minéralisés.]*

Figure 24. - TEM. Section through a cluster composed of mineralized globules (arrows). *[MET. Coupe passant par des agglomérations de globules minéralisés (flèches).]*

Figure 25. - TEM. Demineralized section showing Liesegang's rings within the globules (arrows*). [MET. Coupe de matériel déminéralisé montrant les anneaux de Liesegang dans les globules (flèches).]*

Figure 26. - TEM. Detail of a chondroclast showing two nuclei (n) and microvilli (mv) in the vicinity of mineralized cartilage (mc). *[MET. Chondroclaste où sont visibles deux noyaux et de nombreuses microvillosités au contact du cartilage minéralisé (mc).]*

Figure 27. - TEM. The mineralized cartilage (mc) of a septum is associated with osseous tissue (b). ca: unmineralized cartilage; ch: chondrocyte; ost: osteoid area. *[MET. Le cartilage minéralisé d'un septum (mc) est en contact avec le tissu osseux (b). ca : cartilage non minéralisé ; ch : chondrocyte ; ost : ostéoïde.]*

tion front progresses towards the distal part of the haemal cartilage. The crystals arranged in a radiating manner do not appear to be oriented by the collagen fibrils. This pattern of mineral deposit considered as a spheritic mineralization by Ørvig (1951, 1968) is usually observed in cartilage (for review, see Boyde and Jones, 1983; Hall, 2005). In the centrum, specific interactions occur between the collagen fibrils and the deposition of the crystals; these mineralization processes can be considered as an inotropic mineralization (Ørvig, 1968) usually found in the vertebrate osseous tissues (Moss, 1961). The crystals are often found in the vicinity of the osteoblasts (for review see Karsenty and Wagner, 2002). A definite boundary between the mineralized cartilage and the osseous tissue of the centrum is distinguishable only in the demineralized sections because of the obvious differences of the components of the organic matrix. The thin fibrils, most probably of type II collagen, and aggrecan particles of the haemal cartilage do not intermix with the thick, banded fibrils of type I collagen of the bone of the centrum. In a comprehensive literature review, Beresford (1981) listed about thirty intermediate mineralized tissues named chondroid bone, many considered to be restricted to teleosts (Beresford, 1981; Benjamin, 1988, 1989, 1990; Benjamin and Ralphs, 1991; Benjamin *et al.* 1992; Meunier and François, 1992; Meunier and Huysseune, 1992; Hall, 2005). All these tissues are intermediate between cartilage and bone but also to differ both from cartilage and bone by their histological, histochemical features (for review see Huysseune, 1986, 2000; Francillon-Vieillot *et al.*, 1990; Hall, 1978, 2005). Consistently, ultrastructural data suggest that type I collagen, the main fibrillar collagen type of the osseous tissue and type II collagen, characteristic of cartilage, coexist in chondroid bone (Goret-Nicaise, 1984). We are aware that we consider the clear-cut separation between the mineralized cartilage layer and the osseous tissue without the formation of a chondroid bone only on the basis of ultrastructural analyses. Nevertheless, from a functional point of view, the absence of chondroid bone is consistent with the hypothesis that growth is most probably slow in the haemal arch of adult carps. Even if a chondroid bone that originates by "ossification mixte" (Stephan, 1900; Blanc, 1953) was described in slow-growing parts of the mandibula of the teleost *Trigla capensis* (Haines, 1937), the formation of a chondroid bone is most often associated with a local high growth rate (Schaffer, 1930; Enlow, 1963; Ricqlès, 1975, 1977; Hall, 2005; Gillis *et al.*, 2006). A slow growth rate and a slow mineralization rate of the cartilage proximal thin layer are also suggested by the absence of differentiated hypertrophic chondrocytes. Indeed, the thin mineralized cartilage layer houses randomly distributed chondrocytes that do not show any structural modification such as lost of the discoidal shape, increase of the cell size, and glycogen stores leading to hypertrophy. These data support findings of previous light microscopic data that did not report the presence of hypertrophic chondrocytes in the proximal mineralized cartilage of adult carp abdominal vertebrae (Meunier and Boivin, 1974; Meunier, 1979). A delayed mineralization occurring with a slow rate might prevent the chondrocytes of the proximal cartilage to initiate a regular endochondral ossification process. It was suggested for other tissues such as the thyroid cartilage (Kimpel *et al.*, 1999; Pufe *et al.*, 2004), the mammalian ribs (Kampen *et al.*, 1995), and for pathological tissues such as the epiphyseal plate in metatrophic dwarfism a skeletal dysplasia (Maroteaux *et al.*, 1966; Boden *et al.*, 1987) in which the cartilage mineralizes without identification of chondrocytes becoming hypertrophic.

At the opposite side of the haemal cartilage, the distal part mineralizes similar to endochondral ossification. Nevertheless, some peculiarities are observed in the haemal growth plate. Indeed, it shows an obvious separation between the proliferative and the hypertrophic zones that was not observed in the branchial skeleton of teleosts (Haines, 1934) whose growth plates show characteristics similar to those observed in mammalian endochondral bones. The chondrocytes progress through a continuum from proliferation to differentiation resulting in the production of hypertrophic chondrocytes and undergo degeneration through apoptosis (for review, see Ali, 1983; Olsen *et al.*, 2000; Karaplis, 2002; Hall, 2005). The first stages of mineral deposit resemble those of the proximal layer. Mineralization is spheritic, the individual crystals being organised in a radiating pattern. The crystals form clusters that are only found in the longitudinal septa where the clusters aggregate to form globules. The rings of Liesegang indicate that the individual globules increase through periodic deposit of the mineral (Ørvig, 1951). The globules are arranged in tubes surrounding the hypertrophic chondrocytes as described in the mammalian growth plates (for review, see Boyde and Jones, 1983; Hall, 2005).

We have identified dark chondrocytes at all stages of differentiation in the haemal growth plate. Previously, these cells had been described only in birds and mammals (Wilsman *et al.*, 1981; Farnum and Wilsman, 1989; Erenpreisa and Roach, 1998). Dark chondrocytes undergo degeneration through chondroptosis, a way of cell death that differs from both the classical apoptosis and necrosis; nevertheless it shares features with both (Roach and Clarke, 2000; Roach *et al.*, 2004). As in the growth plate of amniotes, the chondroptotic cells of the haemal cartilage of the carp are also characterised by the excessive development of the rough endoplasmic reticulum and of the Golgi complex. This is consistent with an increase of the synthetic activity observed at the early stages of differentiation of the dark chondrocytes. Dark cells with developed RER and Golgi areas have been identified from the proliterative stage until their destruction at the very distal part of the mineralized cartilage as described in birds and mammals (Erenpreisa and Roach, 1999; Roach *et al.*, 2004). The development of the nuclear material differs from apoptosis since the compacted chromatin is scattered within the nucleus. Chromatin compaction together with a self-destruction of the cytoplasmic components via autolytic vacuoles could be interpreted as an aberrant type of cell death (Erenpreisa and Roach, 1998). However, even if the formation of autophagic vacuoles was considered as a response to stress conditions allowing the cells to survive, we do not know if the chondrocytes that present hallmark features of chondroptosis could be restored in order to be involved in ossification processes (Roach *et al.*, 2004). The light chondrocytes present features of necrosis.

At the distal end of the HGP, the resorption of mineralized cartilage is performed by cells that resemble multinucleated osteoclasts. However, our histological investigations have not allowed us to find whether other cells without distinctive morphological features are associated with the resorption of mineralized cartilage. These cells have to be identified by histochemical techniques showing the presence of the osteoclast marker enzyme tartrate-resistant acid phosphatase (TRAP). Indeed, it is known that in teleosts, flat, mononucleated cells are TRAP-positive and involved in bone resorption (Sire *et al.*, 1990; Witten *et al.*, 2000; Witten *et al.*, 2001). Such flat, mononucleated, TRAP-positive cells, that are thought to be associated with a slow mode of bone remodelling, were found in the neural and haemal arches of the teleost medaka, *Oryzias latipes* (Nemoto *et al.*, 2007).

In contrast, clastic cells implied in resorption of mineralized cartilage were not found in Chondrichthyans (Clement, 1992). The status of chondrichthyan chondrocytes, alive (Moss, 1977; Summers, 2000) or degenerated (Bordat, 1987, 1988) is still a matter of discussion. In chondrichthyan species that are able to form an osseous tissue, mineralized cartilage remains a perennial structure (Kemp and Westrin, 1979; Peignoux-Deville *et al.*, 1982; Bordat, 1987, 1988) where no resorption phenomenon was found (Peignoux-Deville *et al.*, 1982).

Specific microenvironments (Adams and Shapiro, 2002) as well as interactions with muscular, circulatory and neural organs among others (Blair *et al.*, 2002) are considered to influence chondrocyte proliferation, differentiation and mineralization types (Stevens and Williams, 1999; Dean and Summers, 2006). Cyclic mechanical stresses such as intermittent hydrostatic fluid pressure are known to inhibit cartilage growth and ossification thereby maintaining the cartilage phenotype (Wu and Chen, 2000; Koob and Long, 2000; Wu *et al.*, 2001; Carter and Wong, 2003). Even if the persistence of the cartilage phenotype is related to mechanical cues, the latter cannot be the only factor of the persistence of a cartilage sandwiched between two bone tissues. Why the cartilage persists in the abdominal vertebrae and disappears in the other parts of the vertebral column remains unexplained. Analyses of a developmental series are necessary to elucidate the ontogeny of the haemal cartilage within the abdominal vertebrae and further ultrastructural studies are necessary to shed light on the first stages of the mineralization in the haemal arch.

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